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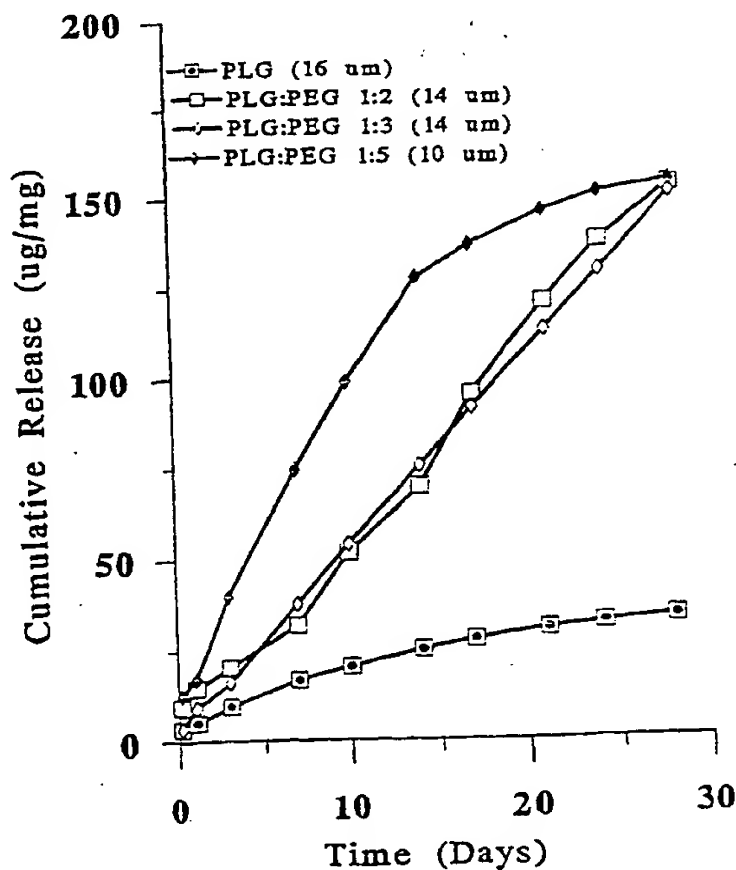please do not report the images to the
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(71) Applicant (*for all designated States except US*): UNIVERSITY OF NOTTINGHAM [GB/GB]; University Park, Nottingham NG7 2RD (GB).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): YEH, Ming-Kung [–/–]; 3F, 10, LN. 78, SunKung RD, Taipei (TW). COOMBES, Alan, Gerald, Arthur [GB/GB]; 21 Stanhome Drive, West Bridgford, Nottingham NG2 7FP (GB). JENKINS, Paul, George [GB/GB]; 9 Ploughman's Way, Tytherington, Macclesfield SK10 2UL (GB). DAVIS, Stanley, Stewart [GB/GB]; 19 Cavendish Crescent North, The Park, Nottingham NG7 1BA (GB).

(74) Agent: BASSETT, Richard; Eric Potter Clarkson, St. Mary's Court, St. Mary's Gate, Nottingham NG1 1LE (GB).

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(54) Title: POLYMER MICROPARTICLES FOR DRUG DELIVERY

(57) Abstract

The invention provides a microparticle comprising a mixture of a biodegradable polymer and a water soluble polymer, and an active agent. Preferred biodegradable polymers are lactide homopolymers or copolymers of lactide and glycolide. Preferred water soluble polymers are PEG (poly(ethylene glycol)) or PEG copolymers. The particles exhibit a linear release profile of active agent. The invention also provides an emulsion/solvent extraction method to make the microparticles. The continuous phase of the secondary emulsion contains an organic solvent which is miscible with the organic solvent in the primary emulsion.

AP5

POLYMER MICROPARTICLES FOR DRUG DELIVERY

The present invention relates to polymer microparticles for administering active agents, and to a method for making such particles.

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BACKGROUND

It is possible to deliver therapeutic agents in the form of drugs and vaccines to the body by a variety of routes that include parenteral and nonparenteral access.
10   The products of biotechnology represent a special class of materials. Today the pharmaceutical scientist is faced with the problem of delivering therapeutically active materials in the form of peptides and proteins, carbohydrates, oliogonucleotides and DNA.

15   Considerable interest exists in the use of colloidal particles for the delivery of therapeutically active materials in the form of proteins and peptides and for vaccine formulation. Various biodegradable polymers have been investigated as therapeutic carriers, including serum albumin beads, polyacryl starch microparticles, polyacrylamide microparticles, poly(butyl-2-cyanocrylate) nanoparticles and polylactide co-glycolide microparticles (Florence *et al*). In
20   the case of albumin and polyacryl starch, antibody responses were induced to the carriers as well as to the specific antigens entrapped therein. Problems of toxicity associated with polyacrylamide and poly(butyl-2-cyanoacrylate) limit the use of these polymers as antigen delivery systems.

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Microparticles based upon resorbable copolymers of polylactide and polyglycolide have been widely investigated for drug delivery (Watts, *et al*) and are now finding increasing application for the delivery of the products of biotechnology (especially peptides and proteins) (Kwong, *et al* and Wang, *et al*). These synthetic polyesters are approved for human use and have a 25-year
30

history of safety. Injected poly(DL-lactide-co-glycolide) (PLG) microparticles exhibit good biocompatibility and induce only a minimal inflammatory response. The lactide copolymers such as PLG are good candidates for the development of controlled release drug systems and vaccines. They biodegrade
5   through hydrolysis of ester linkages to yield the normal body constituents lactic acid and glycolic acid. The degradation rate of lactide copolymers is controlled by various factors including molecular weight, lactide: glycolide ratio and polymer crystallinity and can be varied from several weeks to over a year, thus potentially allowing control over the time and rate of vaccine release. Carriers
10   may therefore be designed to release entrapped antigen at certain intervals after immunization when booster doses are normally administered.

A large number of microencapsulation techniques have been developed using PLG, such as film casting, moulding, spray drying and extrusion, but the most
15   common is the solvent evaporation technique (Fong, *et al.*, and Bodmeier *et al.*,). Although the oil-in water (O/W) emulsion/solvent evaporation technique has been used successfully by several groups to entrap hydrophobic substances such as progesterone, poor encapsulation efficiency results for moderately water-soluble and water soluble drugs due to partition into the aqueous
20   continuous phase (Benita, *et al.*,). This presents a major problem in drug and vaccine delivery.

Protein encapsulation in PLG microparticles has been attempted previously using oil-in oil (O/O) and O/W techniques wherein dried protein is first
25   dispersed in a solution of PLG (Alonso, *et al*). In the O/O technique, the dispersion may be emulsified in silicone oil containing Span 85 as a stabiliser. The addition of petroleum ether subsequently results in solvent extraction and precipitation of the microparticles. Although protein loadings are close to the theoretical maximum, the particle size tends to be large (around 500 μm) and
30   the particle shape irregular. Leelarasamee *et al* described a solvent partitioning

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method which involved slowly injecting a suspension of hydrocortisone in a PLA solution into a stream of mineral oil. In a related method, Wada *et al* encapsulated hydrophilic drugs in lactic acid oligomer microparticles using an acetonitrile/water mixture to form the primary emulsion. This was subsequently

5   emulsified in cottonseed oil and the solvent was removed by heating.

In the O/W approach a suspension of protein in the polymer solution is emulsified with an immiscible aqueous surfactant solution which results in polymer precipitation and hardening of the microparticles. Solvent is removed

10   by evaporation. Microparticles less than 10 μm in size may be produced but the protein is inefficiently encapsulated in the microparticles prepared in the presence of water, due to partition into the aqueous phase. Faster and less reproducible protein release has also been noted when the protein is dispersed as a lyophilised powder in the polymer solution (Alonso, *et al*).

15

In order to improve the loading of water soluble compounds within PLG microparticles Ogawa *et al* used a water-in oil-in water (W/O/W) solvent evaporation technique to entrap a water soluble peptide into PLG microparticles. The W/O/W technique has since become one of the principle

20   methods for encapsulating proteins and peptides (Jeffery, *et al*). In this double emulsion-solvent evaporation approach, an aqueous solution of the protein is emulsified with the polymer solution to form a primary water-in-oil emulsion (W/O). This is subsequently emulsified with an aqueous surfactant solution (W/O/W) to induce polymer precipitation and microparticle hardening and to

25   allow solvent removal by evaporation. The W/O/W method has been used to encapsulate diphtheria toxoid in DL.PLA controlled release microparticles (Singh, *et al.*) and tetanus toxoid in PLA and DL.PLG respectively (Raghuvanshi, *et al)* with the aim of producing single dose vaccines which potentially overcome problems of patient noncompliance, vaccine administration

30   and storage.

A recent publication has described the incorporation of ovalbumin in PLG particles using the W/O/W method (Uchida and Goto. Biol. Pharm. Bull. 17(1994) 1272-1276). The particles were in the size range 1 to 14 μm. The authors commented on the low loading efficiencies (8-20 %). The content of

5   ovalbumin in the microparticles was expected to be from 0.08 to 0.20 %.

Microparticles which are administered subcutaneously either remain in the subcutaneous tissue or are phagocytosed depending on their size (Visscher, *et al*). The encapsulation of antigens in microparticles less than 10 μm is of

10   interest for targeting to macrophages (Eldridge, *et al*). Several studies (Eldridge, *et al*., and Jenkins, *et al*., and Jani, *et al*.) have shown that there is also a significant dependency on size for the absorption of microparticles across the gastrointestinal tract after oral delivery, with microparticles of 0.5-1.0 μm being absorbed in the greatest numbers. Thus, the ability to easily control the

15   particle size is desirable.

It has surprisingly been found that microparticles comprising a mixture of a biodegradable polymer and a water soluble polymer achieve a greatly improved loading of active agent and can give a linear release time profile for the active

20   agent.   Furthermore, it has been found that preparation of polymer microparticles using an emulsification/solvent extraction method in which miscible organic solvents are used significantly reduces partitioning of the active agent into the continuous phase of the secondary emulsion, especially when the active agent is water-soluble.

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The present invention therefore provides a microparticle comprising a mixture of a biodegradable polymer and a water soluble polymer, and an active agent. The term microparticle is used herein to include nanoparticles.   The microparticles preferably have a size in the range 10 nm to 200μm.

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The water-soluble polymer is preferably soluble in both water and dichloromethane (DCM).

We use the term "biodegradable polymer" to include polymeric systems which
5   can degrade into low molecular weight compounds which are known to be involved normally in metabolic pa: ·ys. We also use the term to include polymer systems which can be att   d in the biological milieu so that the integrity of the system, and in some cases of the macromolecules themselves, is affected and gives fragments or other degradation by-products which can
10   move away from their site of action, but not necessarily from the body.

The ratio of biodegradable polymer to water-soluble polymer is in the range 99.9:1.0 to 10:90, more preferably 90 10 to 10:90.

15   Suitable biodegradable polymers for producing the microparticles are polyesters such as polylactide, polyglycolide, copolymers of lactide and glycolide, polyhydroxybutyrate, polycaprolactone, copolymers of lactic acid and lactone, copolymers of lactic acid and PEG, copolymers of α-hydroxy acids and α-amino · acids (polydepsipeptides), polyanhydrides, polyorthoesters,
20   polyphosphazenes, copolymers of hydroxybutyrate and hydroxyvalerate, poly(ethylene carbonate), copoly(ethylene carbonate), polyethylene terephthalate or mixtures of these polymers.

The preferred resorbable/biodegradable polymers are lactide homopolymers
25   poly(L-lactide), poly(D,L-lactide), and copolymers of lactide and glycolide such as 50:50 poly(DL lactide co-glycolide)(PLG). The PLG preferably has a molecular weight in the range 5-100kD.

While poly(ethylene glycol) (PEG) is the preferred water soluble polymer for
30   mixing with the biodegradable polymer, other suitable water soluble polymers

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include poly(oxyethylene oxide)(PEO), poly(oxyethylene)-poly(oxypropylene) [PEO-PPO] block copolymers such as tri-block PEO-PPO-PEO copolymers (Poloxamers, Pluronics) and tetra-functional block copolymers derived from the sequential addition of propylene oxide and ethylene oxide to ethylene diamine

5   (Poloxamines, Tetronics), copolymers of PEG with poly(lactic acid), oligomers of poly(lactic acid), lactides, copolymers of PEG and amino acids, conjugates of PEG with polysaccharides for example a conjugate produced from 40000 MW dextran and polyoxyethylene-glycol monomethyl ether and others as described by Duval et al. in Carbohydrate Polymers, 15, (1991), 233-242,

10   conjugates of PEG with proteins such as those described by Nucci et al., in Advances in Drug Delivery Review, 6, (1981), 113-151, or with collagen as described by Rhee et al in Poly(ethylene glycol) chemistry. Biotechnical and Biomedical Applications. Ed. J. Milton Harris, Plenum Press (1992) or conjugates of PEG with colony Stimulating Factor (CSF-1) as described by

15   Katre N.V. in The conjugation of proteins with polyethylene glycol and other polymers. Adv. Drug Delivery Reviews, 10, 91-114 (1993). Conjugates of PEG with bioactive agents such as enzymes, vitamins, steroids and drugs such as 5-fluoro-uracil.

20   The molecular weight of PEG is in the range 100-100,000. The molecular weight of PEO is usefully in the range 100,000 to 500,000.

The invention further provides a method of forming polymer microparticles containing an active agent comprising the steps of:

25   a.   forming an aqueous solution, water-in-oil (W/O) emulsion or a suspension of the active agent in a first organic solvent;

            b.   mixing the aqueous solution, W/O emulsion or suspension of the active agent with a polymer solution formed in a second organic solvent;

            c.   mixing the emulsion so formed in step b. with a continuous phase

30   comprising a third organic solvent which is miscible with the first and second

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organic solvents and is not a solvent for the polymer.

The continuous phase causes precipitation of the polymer, extraction of the first and second solvents and hardens the microspheres.

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The microparticles are then harvested, cleaned and stored according to usual methods.

The first and second organic solvents may be the same or different and are
10   preferably selected from dichloromethane (DCM), chloroform, acetone, ethyl acetate, ethyl formate or mixtures thereof.

The dry active agent can be added directly to the solution of polymer in the second organic solvent. Thus the solvent used to prepare the water-in-oil
15   emulsion or suspension of the active agent may already contain the dissolved polymer.

The third organic solvent is preferably a lower alcohol having 1 to 6 carbon atoms and is more preferably methanol or ethanol.

20

The first organic solvent preferably contains a stabiliser. Suitable stabilisers include Sorbitan esters, Span 60 (Sorbitan monostearate), glyceryl monoesters, such as glyceryl monostearate, and nonyl phenol ethoxylates or any other stabiliser which is soluble in the first organic solvent. The continuous phase
25   preferably contains a surfactant such as polyvinyl pyrrolidone (PVP) or any other surfactant which is soluble in the third organic solvent.

The emulsification/solvent evaporation technique for preparation of PLG microparticles is generally based on the use of immiscible liquids to form
30   droplets of polymer solution in a continuous phase which subsequently harden

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to form microparticles by polymer precipitation and solvent removal. An exception is the nanoprecipitation method of Fessi *et al.* in which spherical particles may be produced by adding a solution of PLG in acetone to water.

5   In contrast however, the method of the present invention makes use of miscible organic phases for example DCM and methanol, to form microparticles. This method has been found to result in significantly higher entrapment of active agent and greatly reduces the partitioning of the agent into the continuous phase.

10

The term "active agent" is used herein to include any agent which it may be desired to administer to the human or animal body for any purpose, including therapeutic, pharmaceutical, pharmacological, diagnostic, cosmetic and prophylactic agents. The term is also used to include any agents which it may

15   be desired to administer to plants by controlled release, such as agrochemicals including herbicides, pesticides and fertilizers.

The active agent is preferably water-soluble, and we define water-soluble herein as referring to a material which is soluble in water to give a solution of at least

20   1 mg/ml.

The active agent is preferably a polypeptide, peptide or protein, a carbohydrate or an oligonucleotide such as DNA.

25   Suitable active agents include growth hormone, insulin, interferons (alpha, beta, gamma), erythropoietin, colony stimulating factors, parathyroid hormone, leutenizing hormone releasing hormone, calcitonin, heparin, somatostatin and various analogues thereof.

30   The active agent may also be an antigen for use in vaccines and these include

polypeptides, proteins, glycoproteins that are obtained from bacterial, viral and parasitic sources or produced by synthetic methods. We use the term antigen to include any material which will cause an antibody reaction of any sort when administered. Such antigens can be administered by injection or to various

5   mucosal sites (nasal, oral, vaginal, rectal, colonic).

Vaccines for the treatment of allergens and for auto immune diseases are well described in the prior art. For example in autoimmune disease it has been suggested that the slow administration of essential factors can be beneficial.

10   Such factors can include insulin for the treatment of diabeter and collagen for treating rheumatoid arthritis.

The microparticles are useful for delivering a wide range of active agents and can be administered by a wide range of routes, depending on the agent to be

15   delivered. The microparticles may be adapted for injection, either intramuscularly, intravenously, subcutaneously, intraarticularly or intraperitoneally. The microparticles may be adapted for administration to the dermal or epidermal layer of the skin by injection or needleless injector system. The microparticles may also be adapted for administration to mucosa such as

20   the nose, the gastrointestinal tract, the colon, vagina or rectum, or administered to the eye.

The microparticles preferably have a size in the range 10 nm to $200\mu m$. The size chosen for a particular microparticle will depend on the active agent to be

25   delivered, and the intended route of administration.

For oral delivery, particles are conveniently in the size range $0.5-5.0\mu m$. For subcutaneous delivery, a suitable size is $< 100\mu m$. Microparticles for extravascular delivery to the spleen, liver and bone marrow preferably have a

30   size of < 100 nm.

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Microparticles for parenteral delivery conveniently have a size of $< 200 \mu$m, preferably $< 150 \mu$m. Microparticles adapted for intra-arterial chemo-embolisation therapy are preferably of a size up to 100μm and microparticles for targeting an agent to the lung capillaries conveniently have a size in the range of 7μm.

The desired particle size can be obtained by varying the process parameters in manners well known to those skilled in the art. For example changing the particular polymer type used and its molecular weight will change the particle size, an increase in polymer molecular weight generally increasing the particle size. Increasing the polymer concentration also increases particle size.

The microparticles of the invention provide a controlled release system which is useful for delivery of a wide range of active agents. A particularly significant advantage has been found with the particles of the invention, namely that a linear or zero order release profile of the active agent can be achieved. Such a release profile is particularly advantageous for the controlled release of certain bioactive agents such as proteins.

In addition, the use of the mixture of biodegradable and water-soluble polymers in the microparticles of the invention has been found to allow a significant increase in the amount of active agent incorporated into the particles, whilst still giving a linear release profile. This combination of properties has not been achieved with prior art particles comprising only biodegradable polymers.

The protein content of prior art resorbable PLG microparticles produced by the conventional emulsification/solvent evaporation (W/O/W) method can be increased by increasing the amount of protein in the primary emulsion. However, a significant "burst" release of protein occurs in the first 24 hours of release testing due to surface location of protein. In addition the release

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pattern is often non linear, being characterised by a rapid release phase, (the "burst effect", followed by a lag phase when little or no protein is released, followed by a steady rate of release (Cohen et al., in Pharmaceutical Research, 8, 1991, 713-720 (1991)). In other instances, the cumulative amount of protein released from resorbable microparticles shows a linear relationship with the square root of time, signifying a process controlled by diffusional release of protein through a network of water filled channels (Hora et al. in Pharmaceutical Research, 7, 1190-1194 (1990)).

The microparticles of the invention do not suffer from a significant burst release of active agent followed by a lag period when little or no release is seen. Instead, the microparticles of the invention have reduced this problem and are found to give a linear release of active agent.

In the preferred embodiment, since PEG is readily soluble in water, the resorption rate of PLG:PEG blended microparticles would be expected to be substantially modified relative to the unblended PLG system due to PEG leaching from the microparticles to leave a highly porous matrix. Fine control over protein release rate is feasible by appropriate changes in PEG characteristics and content.

The method of the invention has also been found to help in achieving an increased loading of active agent in the particles due to the third organic solvent used in the continuous phase. Additionally, the inclusion of a stabiliser such as Span 60 in the first organic solvent has been found to contribute to the reduction of the "burst" effect found with the microparticles of the invention.

In a preferred embodiment, microparticles containing a mixture of PLG with PEG, made by a method using methanol as the third organic solvent, were found to achieve a significant increase in loading of the active protein agent and

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to exhibit a zero order release pattern of protein over 30 days during *in vitro* incubation at 37°C.

Preferred embodiments of the invention will now be illustrated in the following
5   examples and with reference to the accompanying drawings in which:

figure 1, shows the cumulative release profiles of OVA from PLG microparticles prepared using PLG:PEG solution;

figure 2 shows the cumulative release profiles of OVA from PLG
10   microparticles prepared using PLG:Pluronic solution; and

figure 3 shows the cumulative release profiles of insulin from PLG microparticles prepared using PLG:PEG solution.

Materials:

15

50:50 poly(DL-lactide-co-glycolide), (molecular weight 9000, RG503), 75:25 poly(DL-lactide-co-glycolide), (molecular weight 17,000, RG755), 85:15 poly(DL-lactide-co-glycolide), (molecular weight 54,000, RG858), poly(D,L-lactide):(PLC, molecular weight 332,000, R208), were supplied by Boehringer
20   Ingelheim, Ingelheim, Germany. Polyvinyl alcohol (PVA) (13-23000, 87-89% hydrolyzed), and poly(vinyl pyrrolidone) (PVP) (MW 40,000) were obtained from Aldrich Chemical Co. Inc., Dorset. U.K. Dichloromethane (DCM), and methanol (HPLC grade) were supplied by Fisons, Loughborough, U.K. Ovalbumin (OVA) (grade V), Insulin, leutinising hormone releasing hormone
25   (LHRH), polyethylene glycol (MW 8000) and Span 60 were obtained from Sigma Chemical Co., Dorset. U.K. Pluronic poly ethylene oxide-polypropylene oxide block copolymers L44, L121, L122, L123 and F127 were obtained from BASF Co., Parsippany. N.J. U.S.A.. All materials were used as supplied.

30   The formulations described in the following examples were all prepared using

the 50:50 PLG copolymer (RG 503) unless otherwise specified..

Example 1. Basic formulation of microparticles

5   A solution of Span 60 in DCM (2 ml, 0.5% w/v) was emulsified with an ovalbumin (OVA) aqueous solution (1 ml, 30 mg/ml) using a Silverson homogeniser (Silverson machines, Chesham Bucks U.K) to provide the primary emulsion. The emulsion was then mixed at high speed for 2 minutes with 5 ml polymer solution (6%w/v PLG in dichloromethane) and emulsified

10   for 4 minutes with a continuous phase solution, methanol (20 ml), containing 10%w/v PVP as an emulsion stabilizer. The resulting W/O/O emulsion was stirred for 3-4 hours under ambient conditions to extract DCM. The microparticles were cleaned by centrifuging and resuspension in distilled water a total of three times and then freeze dried. The final product was stored in a

15   desiccator below 4 °C.

Example 2. The effect of concentration of Span in the primary emulsion

       A solution of Span 60 in DCM (2 ml) (0.1to 20.0% w/v) was emulsified with

20   an OVA aqueous solution (1 ml, 30 mg/ml) using a Silverson homogeniser to provide a primary emulsion. The resulting emulsion was then emulsified at high speed with polymer solution (6% w/v PLG in DCM) and emulsified with a continuous phase solution, methanol, containing 10 % w/v PVP as an emulsion stabilizer. The resulting w/o/o emulsion was stirred for 3-4 hours under

25   ambient conditions to extract DCM. The microparticles were cleaned, freeze dried and stored as described above.

       Determination of OVA Loading of Microparticles : 3-5 mg of freeze dried microparticles, accurately weighed, were treated with 1.0ml, 0.1M NaOH

30   containing 5%w/v SDS by shaking overnight on a Ika Vibrax -VXR shaker.

The sample was centrifuged and a BCA protein microassay was used to determine the OVA concentration in the supernatant against a series of OVA standards prepared in 0.1M NaOH containing 5%w/v SDS. Each sample was assayed in duplicate.

5

Particle Size : Particles were sized by laser diffractometry using a Malvern 2600D laser sizer. Average particle size was expressed as volume mean diameter (vmd) in μm.

10   Span in the microparticle formulation has been found to be effective in reducing the burst effect of surface protein, and influences particle size and protein payload (Table 1). When Span 60 was used as a stabiliser in the primary emulsion, the protein entrapment increased with increasing concentration of surfactant, to a peak value of approximately 16% OVA at 3 % Span

15   concentration then decreased with increasing surfactant concentration from 3 to 20 %. The mean particle size was between 10 and 20 μm, and the smallest particles were obtained in the absence of Span 60.

Table 1.   Investigation of the effect of Span 60 concentration in the primary
20   emulsion.

Span Conc.%.	Protein Entrapment %	Entrapment Efficiency %	Particle size (μm) vmd
0	10.7	90.2	9.3
0.5	10.4	89.3	12.6
1.0	11.5	82.7	18.2
2.0	12.2	93.5	16.3
3.0	15.8	98.9	16.9
5.0	12.6	87.9	15.1
10.0	11.3	78.8	10.9

Example 3. The effect of PLG/PEG 8000 solution ratio

5   A solution of Span 60 (3.0 %, 2 ml) in DCM was emulsified with an OVA aqueous solution (1 ml, 30 mg/ml) to provide the primary emulsion. The resulting w/o emulsion was then emulsified at high speed with polymer solution (6% w/v) consisting of PLG blended with different ratios of PEG 8000 in DCM. The emulsion was then mixed with a continuous phase solution, methanol, containing 10 % w/v PVP as an emulsion stabilizer. The resulting

10 w/o/o emulsion was stirred for 3-4 hours under ambient conditions to extract DCM. The microparticles were cleaned, freeze dried and stored as described above.

With 3% Span 60 as a surfactant in the primary emulsion, a clear relationship

15 was found between protein entrapment and PLG/ PEG 8000 solution ratios (Table 2). The 1:3 solution ratio resulted in the highest protein entrapment (72 %). The protein entrapment (%w/w) increased with increasing solution ratio from 0 to 1:3 and then decreased with increasing solution ratio from 1:4 to 1:5. The mean particle size varied between 7 and 18 μm, depending on PLG : PEG

20 solution ratio and the smallest particles (6.6 μm) were obtained using a polymer solution ratio of 1:1.

Table 2.   The effect of   PLG/PEG 8000 solution blend ratio on microparticle size and protein loading

25

PLG/ PEG 8000	Protein Entrapment %	Entrapment Efficiency %	Particle size (μm) vmd
1:0	16.3	94.3	18.6
1:1	43.1	83.4	6.6
1:2	52.8	87.5	18.9

1:3	72.3	89.8	14.7
1:4	39.2	85.7	10.7
1:5	36.8	91.5	11.1

5

Example 4. The effect of the stabiliser concentration in the continuous phase

A solution of Span 60 (0.5 %, 2 ml) in DCM was emulsified with an OVA aqueous solution (1 ml, 30 mg/ml) to provide the primary emulsion. The resulting emulsion was then emulsified at high speed with polymer solution (6% w/v 1/5 PLG/ PEG 8000 in DCM) and emulsified with a continuous phase solution, methanol, containing from 5 to 25 % w/v PVP as an emulsion stabilizer. The resulting w/o/o emulsion was stirred for 3-4 hours under ambient conditions to extract DCM. The microparticles were cleaned, freeze dried and stored as described above.

The mean particle size varied between 10 and 20 μm (Table 3). Protein loading remained fairly constant between 30 and 36 % (Table 1). No significant effects of PVP concentration between 5 and 25% on OVA entrapment are evident.

Table 3.   The effect of PVP concentration in the continuous phase on microparticles size and protein loading

PVP %	Protein Entrapment %	Entrapment Efficiency %	Particle size (μm) vmd
5	36.1	83.1	19.7
10	33.1	92.5	17.7
15	30.7	96.6	16.8
20	32.1	95.4	18.5

25	34.9	87.7	10.c

Example 5. The effect of volume of reagents on microparticle characteristics produced using PLG/PEG solutions

The effect on microparticle characteristics of increasing the volume of polymer solution. The volume ratio of OVA/Span/polymer solution/continuous phase (1/2/5/20) was kept constant. A solution of Span 60 (0.5 %) in DCM (2, 4 and 8 ml) was emulsified with an aqueous OVA solution 30 mg/ml, (1, 2 and 4 ml). The resulting emulsion was then mixed at high speed with the polymer solution (6% w/v PLG/ PEG 8000 : 1/5 in DCM) (5, 10, 20 ml) and emulsified with a continuous phase solution (20, 40, 80 ml), methanol, containing 15 % w/v PVP as an emulsion stabilizer. The resulting w/o/o emulsion was stirred for 3-4 hours under ambient conditions to extract DCM. The microparticles were cleaned, freeze dried and stored as described above.

The effect on microparticle characteristics of increasing the volume of polymer solution (at constant volume ratio of OVA/Span/polymer solution/continuous solution/(1/2/5/20)) is shown in Table 4. The protein entrapment was found to increase with increasing volume from 31.6 %w/w to 47.8 %w/w and the particle size decreased with increasing volume from 16.7 to 2.1 μm.

Table 4.   The effect of polymer solution volume on microparticle size and protein loading

Volume of Polymer solution	Protein Entrapment %	Entrapment Efficiency %	Particle size (μm) vmd
5	31.6	94.6	16.7

| 10 | 39.2 | 96.5 | 8.6 |
| 20 | 47.8 | 98.3 | 2.1 |

Example 6. Protein release microparticles

In-vitro Release of Ovalbumin from Microparticles : A series of tubes, each containing approximately 20 mg freeze-dried microparticles (accurately weighed) and dispersed in 2.0 ml PBS, were retained in a water-bath at 37° C with occasional shaking. Periodically, the microparticle samples were centrifuged (3,800 rpm 5 minutes), the supernatant was removed and the protein content of the supernatant was analyzed using a BCA protein assay. Fresh PBS was added to the microparticles and incubation was continued. Release profiles were calculated both in terms of cumulative release (%) with incubation time and μg OVA/mg of microparticles with incubation time.

The protein release characteristics from microparticles produced by the method of the invention are shown in Figure 1. Cumulative release amount (μg/mg) vs. time relationships are shown. It can be seen that the release pattern follws a zero order release profile. The blended PLG/PEG microparticles prepared according to the invention result in at least a four fold increase in protein release compared with PLG microparticles, produced by the standard methods described in the prior act (Wang, *et al*).

Example 7. Microparticles prepared from blended solutions of lactide polymers and PEG

The effect of lactide polymer type on microparticle characteristics

OVA-loaded microparticles were prepared according to the method described

in Example 3 but using polymers of different lactide:glycolide ratio in a 1:2 blend with PEG. The microparticle characteristics are shown in Table 5. The maximum protein loading level of around 40 % was achieved using poly(D,L-lactide) polymer with PEG 8000. The smallest particle size was obtained using 5   75:25 PLG. Thus it can be seen that the use of different biodegradable polymers in the microparticles formulations as the slow resorbing phase can allow variation of protein loading and microparticle size.

Table 5.   Investigation of the effect of lactide polymer type on particle
10   characteristics

PLG Type*	Protein Entrapment %	Entrapment Efficiency %	Particle size (μm)		
			vmd	d(90)	d(10)
50:50	26.6	95.6	10.6	19.4	2.8
75:25	21.8	81.3	7.7	12.9	2.4
85:15	28.1	99.8	11.8	28.0	1.8
!00:0	40.3	95.2	13 6	33.8	2.4

*: lactide/glycolide ratio.

Average volume mean diameter (vmd): Particle diameter d(90): 90 % below
20   this range; Particle diameter d(10): 10 % below this range.

Example 8. The Effect of PLG:Pluronic 127 Solution Composition on Microparticle Characteristics

25   A solution of Span 60 (2 ml, 0.5 % w/v) in DCM was emulsified with an OVA aqueous solution (1 ml, 30 mg/ml) using a Silverson homogeniser to produce a primary emulsion. The resulting emulsion was then mixed at high speed with a 6% (w/v) polymer solution produced by co-dissolving PLG and Pluronic

F127 in DCM in various ratios : 3:1, 1:1, 1:2 and 1:3. The resulting w/o emulsion was mixed with a continuous phase solution. methanol. containing 15 % w/v PVP as an emulsion stabilizer and the resulting w/o/o emulsion was stirred with a magnetic stirrer for 3-4 hours under ambient conditions to extract
5   DCM. The microparticles were cleaned, freeze dried and stored as described in Example 1.

Samples are designated in the text in terms of the ratio of PLG to Pluronic in the starting polymer solution.
10

The effect of PLG:Pluronic F127 solution composition on microparticle characteristics is shown in Table 6. Blending of Pluronic F127 with PLG results in an improvement of protein loading relative to PLG microparticles. The maximum loading level achieved of around 30 % (1:2 PLG:Pluronic F127)
15   is almost twice that obtained with PLG (16 %) in similar size microparticles. No distinct relationship between protein entrapment and PLG:Pluronic F127 ratio is apparent (Table 6). The microparticle size is seen to decrease with increasing Pluronic F127 ratio in the starting solution from 17.4 μm (3:1) to 6.4 μm (1:3). PLG:Pluronic F127 ratios above 1:3 did not result in ·
20   microparticle formation.

Table 6.   The effect of PLG:Pluronic F127 solution composition on microparticle characteristics

PLG Pluronic ratio	Protein Entrapment %	Entrapment Efficiency %	Particle size (μm)		
			vmd	d(90)	d(10)
1:0	16.3	94.3	18.6	45.7	2.1
	22.4	99.5	17.7	42.7	1.9
3:1	21.8	99.8	17.0	40.7	1.9

	17.5	100	11.9	26.8	2.0
1:1	18.3	99.9	11.9	25.8	2.2
	29.7	99.1	9.51	25.8	1.9
1:2	28.9	98.9	9.45	24.3	2.0
	27.8	96.1	6.4	14.8	1.5
1:3	28.5	95.7	6.4	15.0	1.6

Average volume mean diameter (vmd); Particle diameter d(90): 90 % below this range; Particle diameter d(10): 10 % below this range.

Results tabulated correspond to 2 separate batches of microparticles.

Example 9. The Effect of Reagent Volume on Microparticle Characteristics

(PLG-Pluronic blend solution)

The volume ratio of OVA/Span/polymer solution/continuous phase solution (1/2/5/20) was kept constant. An OVA aqueous solution (1, 2 or 4 ml) was emulsified with a solution of Span 60 (0.5 % (w/v)) in DCM (2, 4 or 8 ml, respectively). The resulting emulsion was then mixed at high speed with polymer solution (6% w/v, 1:1 PLG : Pluronic F127 in DCM) (5, 10 or 20 ml) and emulsified with a continuous phase solution (20,40 or 80 ml), methanol, containing 15 % w/v PVP as an emulsion stabilizer. The resulting w/o/o emulsion was stirred for 3-4 hours under ambient conditions to extract DCM. The microparticles were cleaned, freeze dried and stored as described in Example 1.

Protein entrapment was found to be unaffected by increasing the volume of reagents at constant volume ratio (Table 7). The microparticle size tends to decrease with an increase in reagent volume and the particle size distribution

21

is improved.

The small 3-4 μm microparticles produced by increasing the volume of reagents are potentially suitable for intravenous administration and oral vaccine formulations. In the latter case, microparticles interaction with the Peyer's patches of gut associated lymphoid tissue is enhanced if the particle size is less than 5 μm.

Table 7. The effect of reagent volume on microparticle characteristics
(1:1 PLG:Pluronic F127)

Polymer solution volume	Protein Entrapment %	Entrapment Efficiency %	Particle size (μm)		
			vmd	d(90)	d(10)
	17.4	97.2	7.9	12.6	1.6
5	16.9	96.5	8.0	13.1	1.7
	18.1	98.0	3.9	6.9	1.4
10	18.3	100	3.4	6.0	1.4
	15.9	75.4	4.0	7.1	1.5
20	16.4	76.8	4.1	7.4	1.5

Average volume mean diameter (vmd); Particle diameter d(90): 90 % below this range; Particle diameter d(10): 10 % below this range. Results tabulated correspond to 2 separate batches of microparticles.

Example 10.   **Microparticles prepared from blended solutions of lactide polymers and Pluronic**

The effect of lactide polymer type on microparticle characteristics

OVA-loaded microparticles were prepared according to the method described in Example 8 but using polymers of different lactide:glycolide ratios in a 1:1 blend with Pluronic F127.

5   The maximum loading level of approximately 40 % OVA was found to be obtained using 75:25 PLG copolymer (Table 8). The microparticle size was found to increase with increasing lactide content of the copolymer. The use of poly(D,L-lactide) did not result in microparticle formation.

10   Table 8. The effect of lactide polymer type on microparticle characteristics
            (1:1 PLG:Pluronic F127 solution).

PLG Type*	Protein Entrapment %	Entrapment Efficiency %	Particle size (μm)		
			vmd	d(90)	d(10)
50:50	14.1	70.6	7.8	17.4	1.8
75:25	40.5	52.2	10.1	22.4	2.3
85:15	16.3	80.3	19.6	38.5	3.0
100:0	no microparticles formed				

*: lactide/glycolide ratio.

20   Average volume mean diameter (vmd); Particle diameter d(90): 90 % below this range; Particle diameter d(10): 10 % below this range.

Example 11.   Microparticles prepared from blended solutions of PLG and Pluronic

25

The effect of Pluronic Type on microparticle characteristics

The adjuvant effect of Pluronic PEO-PPO copolymers such as Pluronic L121

has been reported by several groups of workers (Hunter. *et al*). Several other preparations using PEO-PPO copolymers with shorter PPO or longer hydrophilic PEO chains also demonstrated adjuvant activity. Thus microparticulate vaccines prepared from blends of PLG and Pluronic may result
5 . in improved adjuvanticity.

OVA-loaded microparticles were prepared according to the method described in example 8 but using different types of Pluronic PEO-PPO copolymers in a 1:2 blend solution with PLG and decreasing the polymer solution concentration
10   to 3%.

The effect of Pluronic type on microparticle characteristics is shown in Table 9. An OVA loading level of around 40 % in similar size microparticles 3.9-6.2 μm was routinely achieved. No distinct relationship between microparticle
15   size and Pluronic types was apparent (Table 9). The high OVA loading may result partly from the use of a lower polymer solution content (ie 3 % rather than 6 %).

Table 9. The effect of Pluronic type on microparticle characteristics
20   (1:2 PLG:Pluronic solution)

Pluronic Type	OVA Entrapment %	Entrapment Efficiency %	Particle size (μm)		
			vmd	d(90)	d(10)
L44	44.2	100	6.2	11.9	1.2
L121	40.3	100	4.5	8.6	1.3
L122	42.9	100	4.8	9.3	1.4
L123	41.2	100	3.9	7.3	1.2
F127	45.9	100	4.8	9.45	1.4

24

Average volume mean diameter (vmd); Particle diameter d(90): 90 % below this range; Particle diameter d(10): 10 % below this range.

Example 12.   **OVA release from microparticles prepared from PLG:Pluronic blended solutions**

The cumulative release of OVA from various microparticle formulations is plotted vs time in Figures 3 and 4. The change in release pattern and release amount obtained by blending PLG with Pluronic F127 in solution to produce the carrier matrix is apparent. The cumulative release amounts for 1:2 and 1:3 PLG:Pluronic microparticles after one month incubation in PBS at 37°C are similar, amounting to 100 μg OVA/mg microparticles and 110 μg OVA/mg microparticles respectively. The results of curve fitting programmes (PCNONLIN) suggest that protein release from the 1:2 PLG:Pluronic and 1:3 PLG:Pluronic systems conforms to the Higuchi model (release amount dependence on the square root of time). The diffusion rate constants (D) are respectively 19.8 and 20.4 (μg/mg.day$^{0.5}$).

In the case of 3:1 and 1:1 PLG:Pluronic microparticles, the protein release pattern is similar and appears to be following a linear, zero order release profile with a release rate constant (k_o) of 1.3 and 0.97 (μg/mg.day), respectively.

The rapid and efficient protein delivery which characterises the PLG:Pluronic microparticles is expected to be facilitated by Pluronic modified internal surfaces within the carrier which would modulate protein/polymer interactions. The properties of the microparticle matrix such as the propensity for porosity development and the pattern of drug release are expected to be controllable by adjusting the amount and the molecular characteristics of the Pluronic copolymer incorporated in the starting solution. The protein release rate and

25

cumulative amount released is expected to increase with increasing amounts of
hydrophilic Pluronic copolymers in the blend and with decreasing molecular
weight of the Pluronic copolymers.

5   **Example 13.**   **Insulin-loaded microparticles**

Preparation

A solution of Span 60 (2 ml, 0.5 % w/v) in DCM was emulsified with an
10   aqueous insulin solution (1 ml, 50 mg/ml) to provide a primary emulsion. The
resulting emulsion was then mixed at high speed with a 5 ml of 6% (w/v)
polymer solution produced by co-dissolving PLG and PEG 8000 in DCM in a
ratio : 1:2. The resulting w/o emulsion was mixed with 20 ml of continuous
phase solution, methanol, containing 10 % w/v PVP as an emulsion stabilizer.
15   The resulting w/o/o emulsion was stirred with a magnetic stirrer for 3-4 hours
under ambient conditions to extract DCM. The microparticles were cleaned by
centrifuging and resuspension in distilled water a total of three times and then
freeze dried. The final product was stored in a desiccator below 4 °C.

20   **Determination of insulin loading of microparticles by HPLC**

Assay procedure : The chromatographic system consisted of a Lichrospher
100 RP-18 5 μm particle diameter (Merck) (0.46 x 15 cm) column, a LKB
2150 HPLC solvent delivery pump, a Gilson dilutor model 401 sample injector
25   and a LKB 2152 HPLC controller. Peak detection was by UV absorbency at
220 nm using a LKB 2151 variable wavelength detector. Quantification (by
peak area) and recording of chromatograms was accomplished using a HP
3394A integrator.

30   The mobile phase consisted of acetonitrile : water 32 : 68, with 0.06 % TFA,

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which was degassed with helium prior to use. A flow rate of 2.0 ml/min was utilised at room temperature, which resulted in a retention time for the insulin peak of 6 minutes. The sample injection volume was set at 50 μl and samples were assayed in triplicate.The minimum detectable concentration of insulin was

5 1 μg/ml, and the regression relationship for the calibration curve of insulin peak area vs. concentration (between 1 and 100 μg/ml) was over 0.997.

Extraction of insulin from microspheres: Insulin was extracted from the microparticles by the following process. 10 mg of insulin-loaded microparticles

10 were dissolved in 1ml of NN-dimethylacetamide : 1, methyl-2-pyrrolidinone (1:1 ratio) and added to 1.0 ml of acetonitrile. The mixture was agitated using a mechanical shaker for 3 min. 2 ml of 0.1 N phosphate buffer solution (pH 7.4) were added and the tube contents were centrifuged at 3500 rpm for 10 min. The supernatant was centrifuged again at 13600 rpm for 5 min, then 50

15 μl of this solution was analyzed by HPLC. Each sample was assayed in triplicate and the insulin concentration was determined by comparison with a calibration curve.

In-vitro release of insulin from microparticles: A series of tubes, each

20 containing approximately 20 mg freeze-dried microparticles, accurately weighed, were dispersed in 2.0 ml PBS containing 0.01% of methyl cellulose, and retained in a water-bath at 37° C with occasional shaking. Periodically, the microparticle samples were centrifuged (3,600 rpm 5 minutes), and the supernatant was collected and recentrifuged at 13600 rpm for 5 min. 50 μl of

25 this solution was injected onto the HPLC column. Fresh PBS was added to the microparticles and incubation was continued. Release profiles were calculated both in term of cumulative release (% w/w) with incubation time and cumulative release (μg insulin/mg microparticle) with incubation time.

30 The initial insulin content of microparticles, and the insulin content of

27

microparticles after washing in PBS solution at room temperature for 4 hours to remove surface insulin are shown in Table 10. The initial insulin loading level was approximately 17 % w/w, which was reduced after washing to a loading level of approximately 5 %. Thus a large proportion of the insulin

5   originally associated with the microparticles is surface located.

The cumulative release amount of insulin from washed microparticles after one month incubation in PBS at 37°C was approximately 30 μg insulin/mg microparticles (Figure 3). A significant 'burst effect' of surface located insulin

10   occurred from washed microparticles in the first 3 days of release testing amounting to approximately 47 % of the insulin content. After 3 days, uniform release of insulin occurred with a release rate of 0.6 μg/mg/day.

Table 10.   The insulin content (w/w%) of microparticles prepared from 1:2
15   PLG:PEG blend solution.

Initial Insulin Loading (w/w%)	Retained Insulin Content (w/w%) after washing in PBS
16.8	5.1
16.9	4.9

20

Attemps have been made in the prior art to entrap insulin in microparticles. Kwong *et al*. described the preparation of insulin loaded poly lactic acid particles that were over 100 μm in size. A burst release effect occurred during

25   the first hour at 0° C of more than 50 % of the insulin load.. Slow release of the remaining insulin was the obtained. The duration of action of the particles could be varied from a few hours to several days. The insulin content of these particles described in the prior art was less than 5 % w/w before washing. This

value dropped to 2 % w/w or less after washing.

Example 14.   LHRH-loaded microparticles

5 **Preparation**

A solution of Span 60 (2 ml, 0.5 % w/v) in DCM was emulsified with an aqueous LHRH solution (1 ml, 50 mg/ml) to provide a primary emulsion. The resulting emulsion was then mixed at high speed with a 5 ml of 6% (w/v)

10 polymer solution produced by co-dissolving PLG and PEG 8000 in DCM in a ratio of 1:2. The resulting w/o emulsion was mixed with 20 ml of continuous phase solution, methanol, containing 10 % w/v PVP as an emulsion stabilizer. The resulting w/o/o emulsion was stirred with a magnetic stirrer for 3-4 hours under ambient conditions to extract DCM. The microparticles were cleaned by

15 centrifuging and resuspension in distilled water a total of three times and then freeze dried. The final product was stored in a desiccator below 4 °C.

The assay procedure used for determination of LHRH loading of microparticle is as described in Example 13.

20

The LHRH content of microparticles, and the corresponding microparticle size range are shown in Table 11.

Table 11.   The LHRH content (w/w%) and particle size of microparticles

25   prepared from 1:2 PLG:PEG blend solution.

Application	Protein Entrapment %	Particle size range (μm)
LHRH	27.9	5-10

Example 15.   DNA-loaded microparticles

A solution of Span 60 in DCM (2 ml, 0.5% w/v) was emulsified with 1 ml of
a DNA aqueous solution (1 mg Plasmid PT 7T3, 400 μl water, 200 μl ethanol,
5   400μl TE (Tris/EDTA) pH 8.5). The emulsion was mixed for 2 minutes with
5 ml of polymer solution in DCM (6% w/v, 1:1 PLG:PEG) and emulsified for
4 minutes with a continuous phase solution, methanol (20 ml) containing 10%
w/v PVP as an emulsion stabiliser. The resulting W/O/O emulsion was stirred
for 3-4 hours under ambient conditions to extract DCM. The microparticles
10   were cleaned by centrifuging and resuspension in distilled water, then freeze
dried and stored in a dessicator below 4°C.

Examination of the microparticles using scanning electron microscopy revealed
two populations of roughly spherical microparticles, one having diameters
15   ranging from 10 to 40 μm, the second having a mean diameter of
approximately 100 μm.

Microparticles were treated with chloroform/water to extract DNA and the
DNA was detected by agarose gel electrophoresis. The presence of a band on
20   the gel was revealed by a UV transilluminator confirming the presence of DNA
in the microparticle sample.

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CLAIMS

1.   A microparticle comprising a mixture of a biodegradable polymer and a water soluble polymer, and an active agent.

5

2.   A microparticle according to claim 1 which exhibits an active agent release profile which is linear with time.

3.   A microparticle according to claim 1 or 2 wherein the microparticle has a
10   size in the range 10 nm to 200 μm.

4.   A microparticle according to any one of claims 1 to 3 wherein the water-soluble polymer is poly(ethylene glycol) (PEG), or a block copolymer comprising PEG as one of the blocks.

15

5.   A microparticle according to any one of the preceding claims wherein the biodegradable polymer is a lactide homopolymer or a copolymer of lactide and glycolide.

20   6.   A microparticle according to claim 5 wherein the biodegradable polymer is poly(lactide co-glycolide) with a molecular weight in the range 5-100kD.

7.   A microparticle according to claim 4 wherein the molecular weight of the PEG is in the range 1-100kD.

25

8.   A microparticle according to any one of the preceding claims wherein the active agent is a peptide, polypeptide or a protein.

9.   A microparticle according to any one of Claims 1 to 7 wherein the active
30   agent is DNA, a vaccine an allergen or an antigen.

10. A microparticle according to Claim 8 wherein the active agent is insulin, LHRH or an analogue thereof, a growth hormone, interferon, colony stimulating factor, somatostatin or an analogue thereof.

5   11. A microparticle according to any one of the preceding claims wherein the ratio of the biodegradable polymer to the water-soluble polymer is in the range of 99.9:1.0 to 10:90.

12. A microparticle according to any one of the preceding claims which is 10   suitable for administration by intramuscular, intravenous, subcutaneous, intraarticular or intraperitoneal injection.

13. A microparticle according to any one of Claims 1 to 11 which is suitable for administration to the dermal or epidermal layer of the skin by injection or 15   needleless injector system.

14. A microparticle according to any one of Claims 1 to 11 which is suitable for administration to the eye, nose, oral cavity, gastrointestinal tract or vaginal cavity.
20

15. A microparticle according to any one of Claims 1 to 11 which is suitable for administration to plants.

16. A method of forming polymer microparticles containing an active agent 25   comprising the steps of:

a.   forming an aqueous solution, water-in-oil (W/O) emulsion or a suspension of the active agent in a first organic solvent;

b.   mixing the aqueous solution, W/O emulsion or suspension of the active agent with a polymer solution formed in a second organic solvent;

30   c.   Mixing the emulsion so formed in step b. with a continuous phase

comprising a third organic solvent which is miscible with the first and second organic solvents and is not a solvent for the polymer.

17. A method according to Claim 16 wherein the solvent used to produce the water-in-oil emulsion or the suspension of the active agent contains the dissolved polymer and step b. is omitted.

18. A method according to Claim 16 or 17 wherein the polymer solution comprises a mixture of a biodegradable polymer and a water soluble polymer.

19. A method according to any one of Claims 16 to 18 wherein the third organic solvent is a lower alcohol having 1-6 carbon atoms.

20. A method according to Claim 19 wherein the third organic solvent is methanol.

21. A method according to any one of Claims 16 to 20 wherein the first and second organic solvent are the same or different and are selected from dichloromethane (DCM), chloroform, acetone, ethyl acetate, ethyl fumate or miscible mixtures thereof.

22. A method according to any one of Claims 16 to 21 wherein the first organic solvent contains a stabiliser.

23. A method according to any one of Claims 16 to 22 wherein the continuous phase contains a surfactant.

24. A method of administering an active agent to an animal or human comprising administering parenterally or non-parenterally a microparticle comprising a mixture of a biodegradable polymer and a water-soluble polymer,
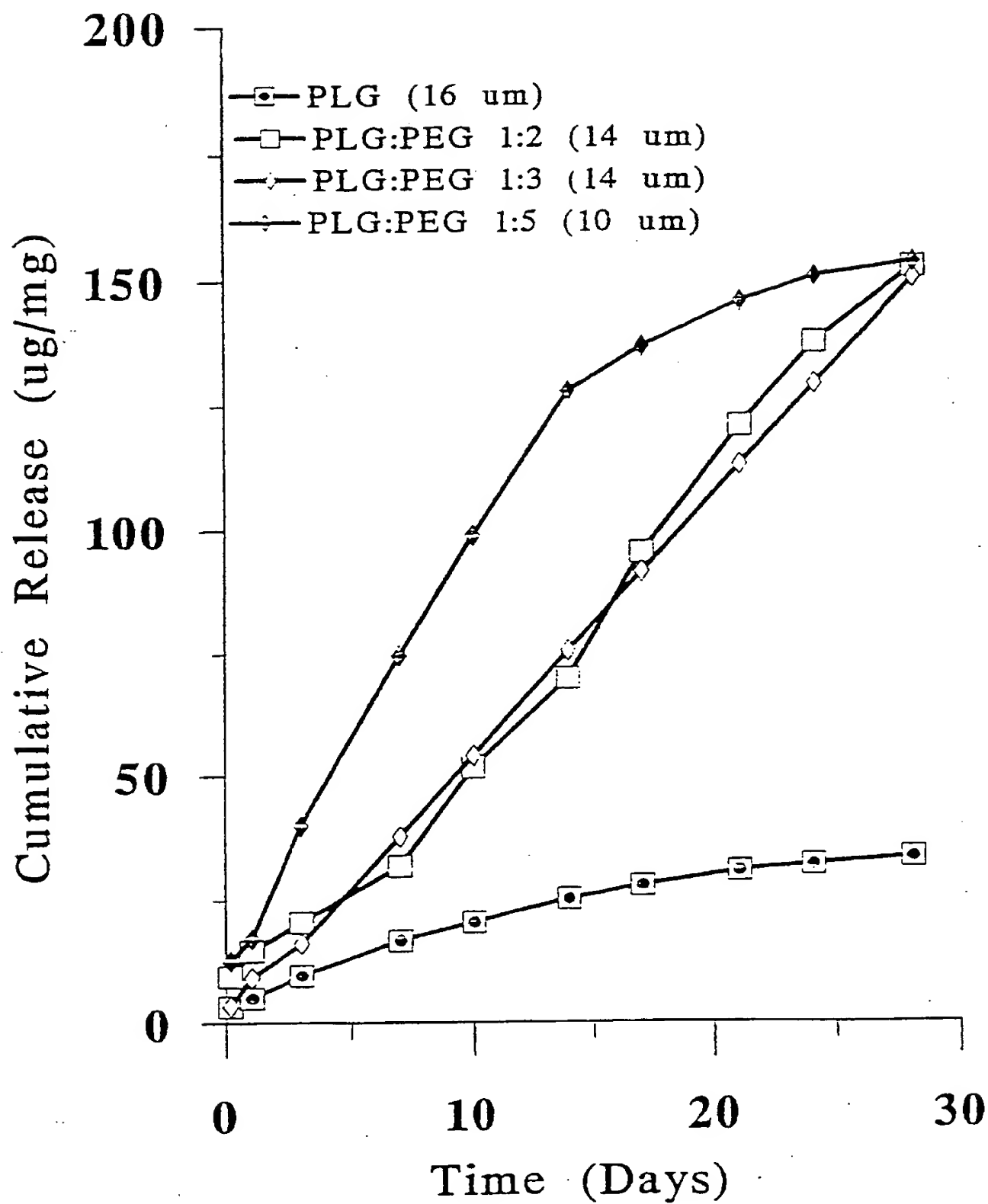
35

and containing the active agent.

FIGURE 1

Time (Days)

1 / 3

FIGURE 2

—⊙—PLG:Pluronic F127 3:1
—☐—PLG:Pluronic F127 1:1
—△—PLG:Pluronic F127 1:2
—△—PLG:Pluronic F127 1:3
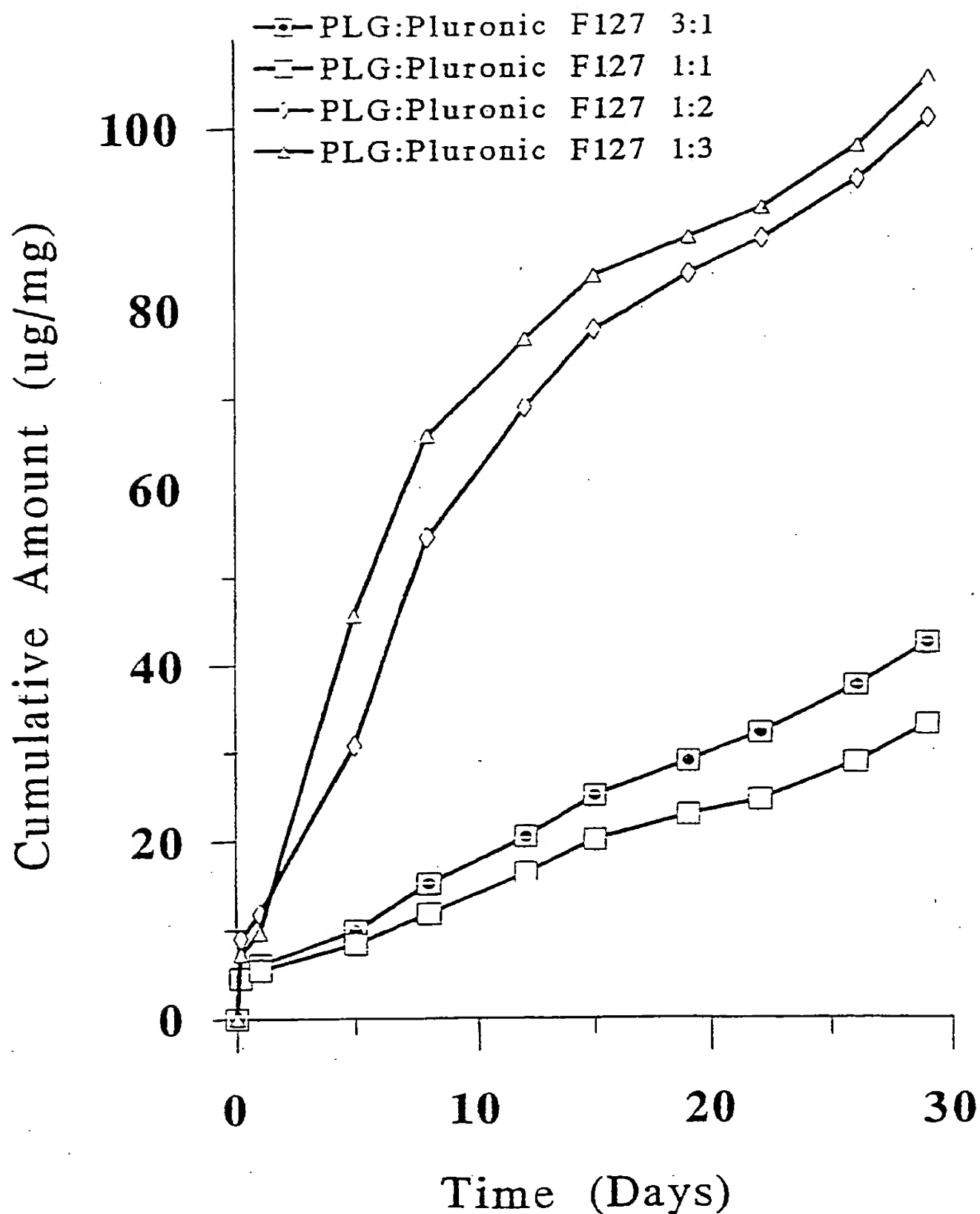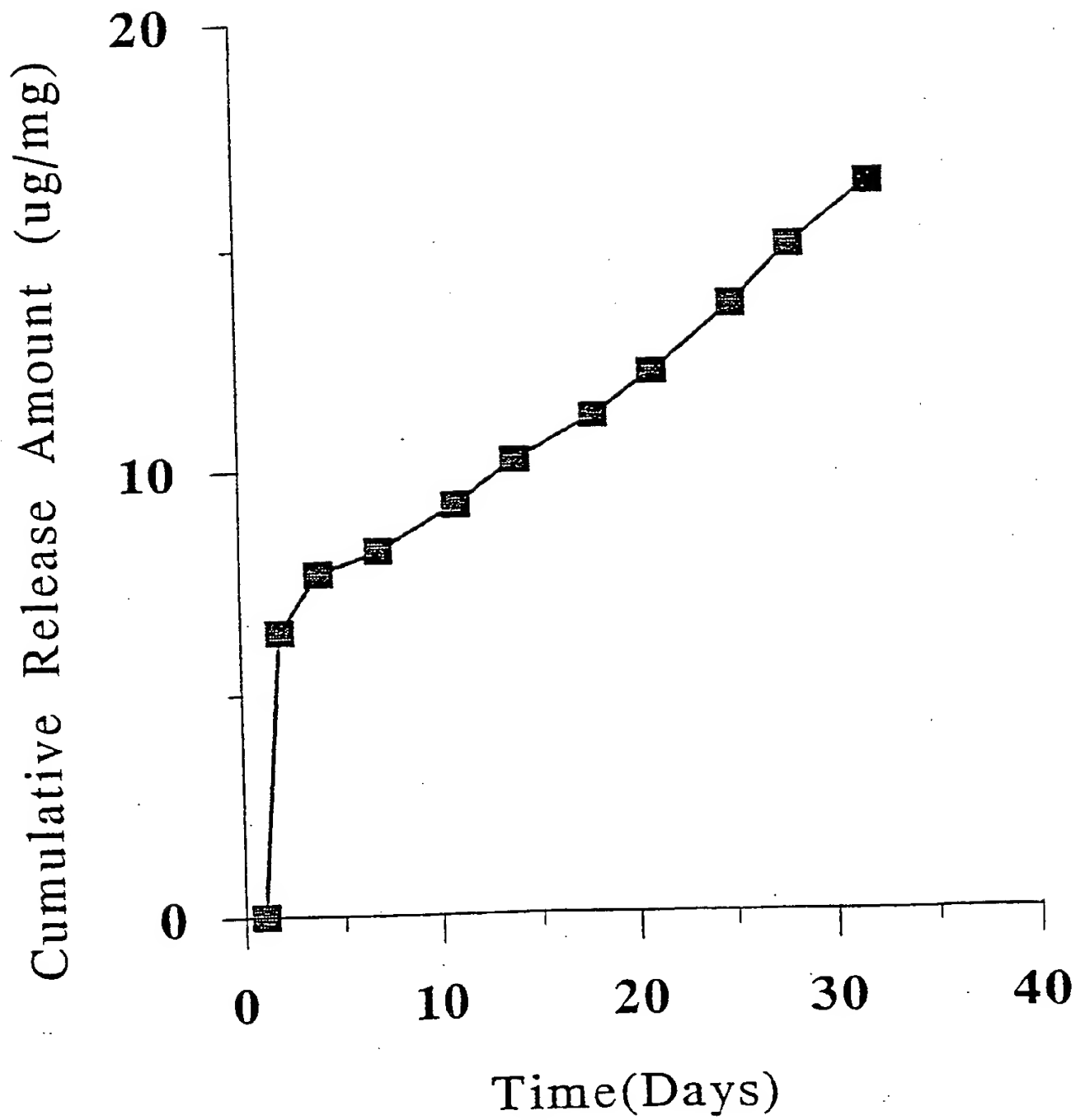
2 / 3

SUBSTITUTE SHEET (RULE 26)

FIGURE 3

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   A61K9/16

According to International Patent Classification (IPC) or to both national c   and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classi: .ıbols)

IPC 6   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	. 'evant to claim No.
X	EP A,0 486 959 (VECTORPHARMA INTERNATIONAL) 27 May 1992 see claims 1,2,4,6,8,10,17 see page 3, line 25 - line 46 see page 4, line 10 - line 32 see page 5, line 1 - line 6 ---	1,3-14
X	DE,A,39 16 020 (WICHERT, BURKHARD, ET AL.) 22 November 1990 see the whole document -----	1-7, 11-14

[ ] Further documents are listed in the continuation of box C.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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2 October 1995	- 6. 10. 95

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0486959	27-05-92	IT-B-	1243390	10-06-94
		JP-A-	4283510	08-10-92
DE-A-3916020	22-11-90	NONE		